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Erythrocyte membrane modifying agents and the inhibition of *Plasmodium falciparum* growth: structure–activity relationships for betulinic acid analogues

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Abstract—The natural triterpene betulinic acid and its analogues (betulinic aldehyde, lupeol, betulin, methyl betulinate and betulinic acid amide) caused concentration-dependent alterations of erythrocyte membrane shape towards stomatocytes or echinocytes according to their hydrogen bonding properties. Thus, the analogues with a functional group having a capacity of donating a hydrogen bond (COOH, CH₂OH, CONH₂) caused formation of echinocytes, whereas those lacking this ability (CH₃, CHO, COOCH₃) induced formation of stomatocytes. Both kinds of erythrocyte alterations were prohibitive with respect to *Plasmodium falciparum* invasion and growth; all compounds were inhibitory with IC₅₀ values in the range 7–28 µM, and the growth inhibition correlated well with the extent of membrane curvature changes assessed by transmission electron microscopy. Erythrocytes pre-loaded with betulinic acid or its analogues and extensively washed in order to remove excess of the chemicals could not serve as hosts for *P. falciparum* parasites. Betulinic acid and congeners can be responsible for in vitro antiplasmodial activity of plant extracts, as shown for *Zataria multiflora* Boiss. (Labiatae) and *Zizyphus vulgaris* Lam. (Rhamnaceae). The activity is evidently due to the incorporation of the compounds into the lipid bilayer of erythrocytes, and may be caused by modifications of cholesterol-rich membrane rafts, recently shown to play an important role in parasite vacuolization. The established link between erythrocyte membrane modifications and antiplasmodial activity may provide a novel target for potential antimalarial drugs.

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1. Introduction

The erythrocyte (red blood cell) membrane is a tri-lamellar composite consisting of a lipid bilayer and an underlying protein network. The latter, consisting mainly of spectrin, supports the lipid bilayer and contributes heavily to the maintenance of shape and elasticity of the cell.¹ Normally, the erythrocytes have a discocytic shape. They are, however, highly deformable cells capable of passing blood capillaries thinner than their diameter. Various agents and conditions cause changes of the erythrocyte cell shape either towards stomatocytic (cup-shaped) or towards echinocytic (spi-

culated) forms.^{2–5} Shape-transformed human erythrocytes exhibit altered membrane permeability⁶ and increased resistance to cell deformation.⁷

Erythrocytes function as host cells during the life cycle of *Plasmodium* parasites, the unicellular protozoans causing malaria, which is one of the most prevalent and disabling human diseases affecting 5–10% of world's population. Erythrocytes are invaded by merozoites, the final stages of the parasite hepatic cycle, leading to the development of a parasitophorous vacuole in which the merozoite matures to the intra-erythrocytic stage (ring stage).⁸ As it grows inside the erythrocyte, the parasite causes extensive modifications of the host cell, including rearrangement of its macromolecules, expression of numerous own proteins, and establishing new

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permeation pathways.^{9–11} These changes result in altered membrane properties of the infected cells, such as changed deformability and susceptibility to haemolysis, and increased cell adhesiveness.^{10–13} Parasitized erythrocytes exhibit altered contents of phosphatidylcholine and phosphatidylethanolamine molecular species in the membrane.¹⁴ Studies indicated that the transport of parasite proteins in the infected erythrocytes is vesicle-mediated,¹⁵ and that membrane oxidation is involved in activation of endogenous channels by the parasite,¹⁶ emphasizing the importance of lipid bilayer composition for the parasite growth.

It may thus be expected that changes in composition, properties and form of the erythrocyte membrane, manifested as rheological changes or as microscopically observable alterations of the cell curvature, can affect the *Plasmodium* parasite invasion and growth. There are indeed various reports to support this.¹⁷ Ovalocytic erythrocytes typical for the Melanesians, which have an altered cytoskeleton, are more resistant to the deformation than normal erythrocytes and less prone to the invasion by merozoites.^{18,19} Merozoites preferentially invade young red blood cells, which are more deformable and have a different membrane composition than mature erythrocytes.²⁰ The inhibition of invasion was observed in resealed erythrocyte ghosts upon chemical or immunochemical cross-linking of spectrin.²¹ Moreover, inhibition of invasion has been observed upon treatment of erythrocytes with membrane-active drugs such as colchicine and vinblastine, but very high (millimolar) concentrations were used.²² Very recently, compounds possibly acting as cholesterol mimics were shown to inhibit proliferation of malaria parasites in vitro in a low-micromolar concentration range.^{23,24} This shows that there is a link between the erythrocyte membrane properties and the ability of erythrocytes to function as hosts for the *Plasmodium* parasites, which could possibly be exploited for a novel antimalarial therapy.

Already early studies linked the erythrocyte membrane cholesterol content with the susceptibility to parasitic invasion in vitro.²⁵ *Plasmodium falciparum* cannot synthesize cholesterol, and malaria infection results in hypocholesterolaemic conditions.²⁶ Recent studies demonstrated the presence of lipid membrane microdomains or rafts, which can be isolated as detergent-resistant membranes and which have an elevated content of cholesterol.^{27–29} It was recently found, that raft-anchored proteins of the erythrocyte host are internalised by *Plasmodium* vacuoles.³⁰ When cholesterol is depleted from the infected erythrocytes, the parasite is expelled from the vacuole,³¹ and *Plasmodium* infection is blocked by cholesterol depletion from the rafts.³² Since lipid rafts apparently play a role in endovacuolation of the parasites and macromolecular trafficking in the infected erythrocytes, and because of the cholesterol demand for production of new generations of merozoites, the infection may be generally affected by compounds altering the cholesterol content or properties of the erythrocyte membrane, for example, by structural analogues of cholesterol. This may provide a general

mechanism by which membrane-active chemicals affect parasite invasion and growth. In the present work, we correlate membrane effects of a series of analogues of betulinic acid, a pentacyclic triterpene having a structure reminiscent of that of cholesterol, with their in vitro inhibition of *P. falciparum* growth. Such correlations are of interest in order to shed light on the mechanism of antiplasmodial activity of membrane modulating compounds.

2. Results

2.1. Betulinic acid as an in vitro antiplasmodial plant constituent

Ethanol extracts of Iranian medicinal plants *Zataria multiflora* Boiss. (Labiatae) and *Zizyphus vulgaris* Lam. (Rhamnaceae) showed in vitro antiplasmodial activity against *P. falciparum* strain 3D7 with $IC_{50} < 12.5 \mu g mL^{-1}$. Bioactivity-directed fractionation of the extracts led to isolation of betulinic acid (**1**, Fig. 1) from both plants. In addition, *Z. vulgaris* yielded betulinic aldehyde (**2**). The compounds were identified by comparison of their 1H and ^{13}C NMR spectroscopic data and optical rotations with those of authentic compounds. IC_{50} values for antiplasmodial activity of purified isolates **1** and **2** were about $6 \mu g mL^{-1}$ ($14 \mu M$). After the compounds were identified as antiplasmodial constituents of the plants, all subsequent experiments were carried out using a commercial sample of **1** and a synthetic sample of **2**.

2.2. Synthesis of betulinic acid analogues

In order to investigate structure–activity relationships for the antiplasmodial effect, several synthetic analogues of **1** were included in the investigation along with lupeol (**3**).²³ The aldehyde **2**, only a small amount of which was isolated from *Z. vulgaris*, was synthesized by oxidation of the commercially available betulin (**4**) with pyridinium dichromate.³³ The oxidation of the primary hydroxy group in **4** proceeded with enough selectivity to afford the required product (**2**) in 20% yield, but concomitant oxidation of the secondary hydroxy group was observed.³³ The ester **5** and the amide **6** were obtained by reaction of betulinic acid pentafluorophenyl ester 3-*O*-trifluoroacetate (**7**) with methoxide ion and ammonia,

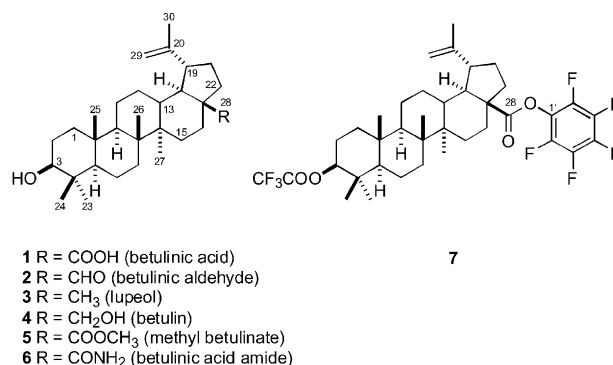


Figure 1. Structures of compounds 1–7.

respectively. The active ester **7** was prepared from **1** and pentafluorophenyl trifluoroacetate.³⁴ Deprotection of the 3-hydroxy group in **7** proceeded simultaneously with the reaction of the pentafluorophenyl ester group. High reactivity usually observed with pentafluorophenyl esters was not shared by **7** and its conversion to **5** and **6** required rather harsh conditions, reflecting a considerable steric hindrance around C-28. Physical and spectroscopic data for **2** and **5** were identical with those reported^{35–37} (the ¹³C NMR spectrum of a compound formulated as **2**, published by Zhong et al.,³⁸ is different particularly for the E-ring carbons, and thus this compound cannot have the structure assigned to it, being probably a stereoisomer of **2**). Compounds **6** and **7** are new.

2.3. In vitro antiplasmodial activity of betulinic acid analogues

Antiplasmodial activity of **1–6** was determined by inhibition of incorporation of [³H]phenylalanine into a chloroquine-sensitive 3D7 strain of *P. falciparum* similarly as previously described.^{23,39} The results are shown in Table 1. The IC₅₀ values observed for the analogues were in a range of 7–28 µM, the parent compound lupeol (**3**) being the least active. Betulin (**4**) exhibited a non-sigmoid (biphasic) dependence of the growth inhibition with concentration (data not shown). Limited solubility of **4** possibly contributed to this effect, but various procedures for stock dilution (sequential dilution of a DMSO stock with growth medium or a parallel addition of the stock solution to obtain the final concentrations in the growth medium directly) had not affected the final result. However, more than 50% growth inhibition was observed at concentrations below 12 µg mL⁻¹ (27 µM).

2.4. Effects of betulinic acid and analogues on erythrocyte membrane

In order to assess effects of **1–6** on the erythrocyte membrane, various concentrations of the compounds were incubated with non-parasitized erythrocytes as in the assay for the antiplasmodial activity. The concentration ranges used were chosen to cover the regions in which the compounds exhibited the antiplasmodial effects (Table 1). After incubation the cells were fixed

with glutaric dialdehyde, dehydrated and embedded in Epon similarly as previously described.²³ Alterations of the cell shape were assessed using transmission electron microscopy. Control cells showed the normal discocytic shape, and DMSO used for preparation of the stock solutions and present in the medium did not affect the cells at concentrations up to 2% (Fig. 2).²³ Erythrocytes incubated with **1–6** showed concentration-dependent changes of the erythrocyte shape. Betulinic acid (**1**), betulin, (**4**) and betulinic acid amide (**6**) induced formation of echinocytes, whereas betulinic aldehyde (**2**) and methyl betulinate (**5**) caused formation of stomatocytes, similarly as lupeol (**3**).²³ A summary of the cell shape changes is given in Table 2. Figure 2 shows concentration dependence of the cell shape alterations caused by **1**, and examples of the effects of **2**, **4** and **6** are given in Figure 3. Increased concentration led to increasingly altered cell shapes, with all cells transformed and some lysis taking place at the highest concentrations used. The stomatocytogenic compounds (**2** and **5**) induced endovesiculation, and the number as well as the size of the vesicles increased with increasing concentration. The vesicles were located mainly along the erythrocyte membrane. The echinocytogenic compounds (**1**, **4** and **6**) caused formation of exovesicles, the amide **6** being the most active. Interestingly, betulin (**4**) caused a progressive echinocyte formation with concomitant formation of small exovesicles at concentrations up to 50 µg mL⁻¹, but the cells incubated with 200 µg mL⁻¹ of **4** showed almost normal cell shape and a smaller number of exovesicles (Fig. 4). Consistent findings were observed using various methods of sample fixation, and thus the observed changes are not artefacts induced by sample preparation. Regardless whether the compounds caused stomatocyte or echinocyte formation, there was a good correlation between the extent of the microscopically observed cell shape changes (Table 2, Figs 2 and 3) and the IC₅₀ values for the antiplasmodial effect (Table 1). The most potent analogue **5** showed detectable cell shape changes at the lowest concentrations.

Table 1. In vitro antiplasmodial activity of betulinic acid and analogues against *Plasmodium falciparum* strain 3D7

Compd	IC ₅₀ ^a	
	µg mL ⁻¹	µM
1	6.3 ± 1.3	13.9 ± 2.9
2	6.2 ± 1.5	14.0 ± 3.5
3	11.8 ± 0.2 ^b	27.7 ± 0.5 ^b
4	< 12 ^c	< 27 ^c
5	3.3 ± 0.9	7.0 ± 2.0
6	6.4 ± 0.6	14.1 ± 1.3
Chloroquine	0.025 ± 0.007	0.048 ± 0.013

^a Concentration necessary to inhibit growth by 50%.

^b From ref 23.

^c Biphasic growth inhibition curve (see text).

Table 2. Erythrocyte shape changes caused by incubation with betulinic acid and analogues determined by transmission electron microscopy^a

Concentration µg mL ⁻¹	Compd					
	1	2	3^b	4	5	6
200	—	—	SS	E1-D	—	—
100	—	—	—	—	—	—
50	E2	S2-SS	SS	E2	—	E3
25	—	—	S1-S2	E2	S2	—
12.5	E2	S2	S1-S2	—	—	E3
6.25	E1	S1	—	E1	S1	E1
3.13	E1-D	S1	D-S1	D	D-S1	E1
1.56	—	—	—	D	D-S1	—
0.78	E1-D	D	—	—	—	D
0.39	—	—	—	D	D-S1-E1	—
0 (control)	D	D	D	D	D	D

^a Only the dominating erythrocyte shapes are listed: D, discocyte; E1, E2, E3, echinocyte type 1, 2 and 3; S1, S2, stomatocyte type 1 and 2; SS, spherostomatocyte (according to the nomenclature of Bessis²); the compounds were not tested at the concentrations for which no shape symbols are given.

^b Used as positive control, cf. ref 23.

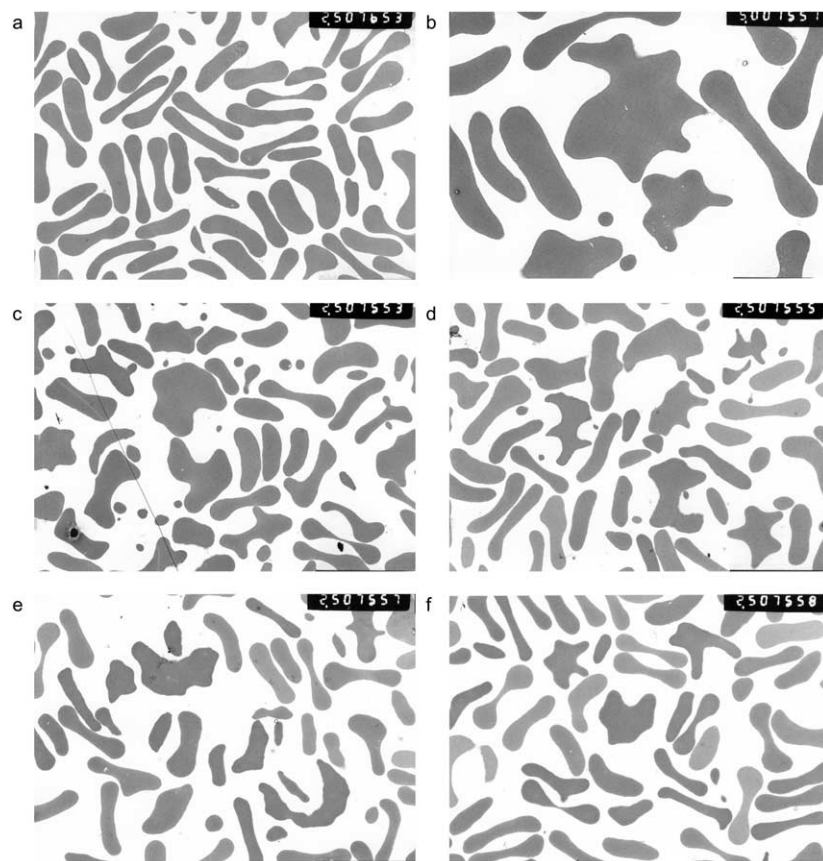


Figure 2. Effect of betulinic acid (**1**) on human erythrocyte shape: (a) Control erythrocytes incubated with 2% DMSO; (b)–(f) erythrocytes treated with $50 \mu\text{g mL}^{-1}$ ($109.5 \mu\text{M}$), $12.5 \mu\text{g mL}^{-1}$ ($27.4 \mu\text{M}$), $6.25 \mu\text{g mL}^{-1}$ ($13.7 \mu\text{M}$), $3.13 \mu\text{g mL}^{-1}$ ($6.9 \mu\text{M}$) and $0.78 \mu\text{g mL}^{-1}$ ($1.7 \mu\text{M}$) of **1**, respectively. Micrographs were obtained with a transmission electron microscope at 1250 times magnification except for (b), which was obtained at 2500 times magnification.

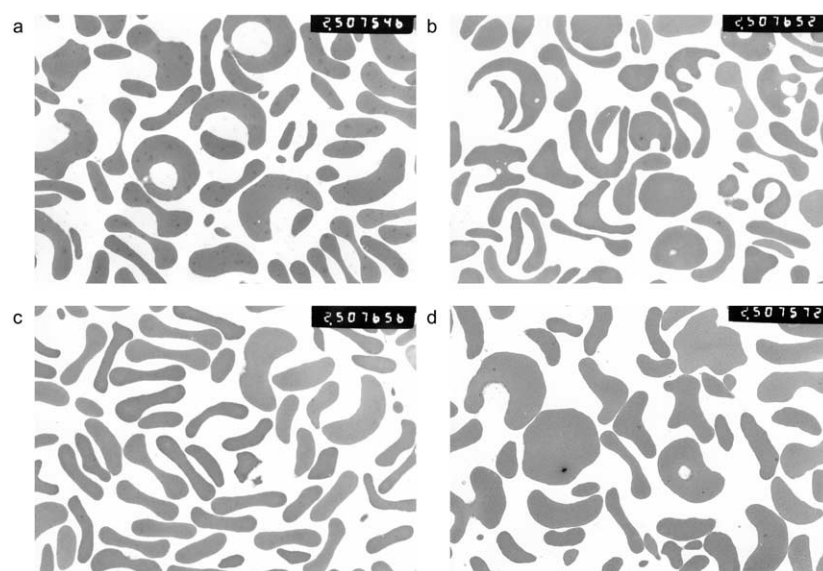


Figure 3. Effect of betulinic aldehyde (**2**), lupeol (**3**), methyl betulinate (**5**), and betulinic acid amide (**6**) on human erythrocyte shape: (a) erythrocytes incubated with $6.25 \mu\text{g mL}^{-1}$ ($14.2 \mu\text{M}$) of **2**; (b) erythrocytes incubated with $12.5 \mu\text{g mL}^{-1}$ ($29.3 \mu\text{M}$) of **3**; (c) erythrocytes incubated with $3.13 \mu\text{g mL}^{-1}$ ($6.7 \mu\text{M}$) of **5**; (d) erythrocytes incubated with $6.25 \mu\text{g mL}^{-1}$ ($13.7 \mu\text{M}$) of **6**. Micrographs were obtained with a transmission electron microscope at 1250 times magnification.

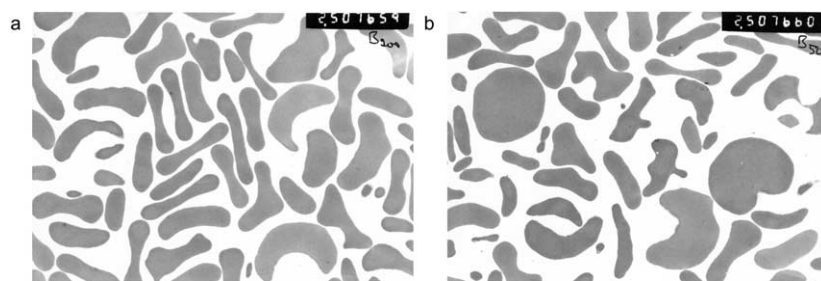


Figure 4. Effect of betulin (**4**) on human erythrocyte shape: (a) erythrocytes incubated with $200 \mu\text{g mL}^{-1}$ ($451.7 \mu\text{M}$) of **4**; (b) erythrocytes incubated with $50 \mu\text{g mL}^{-1}$ ($112.9 \mu\text{M}$) of **4**. Micrographs were obtained with a transmission electron microscope at 1250 times magnification.

2.5. Effect of pre-treatment of erythrocytes with betulinic acid, betulinic aldehyde and betulin on *P. falciparum* growth

After the erythrocytes were pre-treated with betulinic acid (**1**), betulinic aldehyde (**2**) or betulin (**4**), extensively washed in order to remove the chemicals from the medium, and inoculated with a *P. falciparum* culture, strong growth impairment was observed. Thus, parasitized erythrocytes (parasitemia 5%) were mixed with erythrocytes pre-incubated for 24 h with **1** (6.25^{-1} or $50 \mu\text{g mL}^{-1}$), **2** (6.25 or $50 \mu\text{g mL}^{-1}$) or **4** (6.25 , 50 or $200 \mu\text{g mL}^{-1}$), and growth of the parasites in the cultures was followed as a function of time (Fig. 5). During the first 4 days of the experiment, parasites of all stages could be observed in the cultures. After additional 2 days most of the parasites were extracellular, with an excess of ruptured schizonts and merozoites. At day 8, virtually no live parasites were observed in any of the cultures employing pre-treated erythrocytes. At the same time, the control culture exhibited a normal growth pattern, reaching 5% parasitemia on day 4, when the parasites were sub-cultured and then continued to grow normally. Erythrocytes used for the control culture were incubated and washed in the same way as the test cultures, but without the chemicals present in the incubation medium.

3. Discussion

Bioassay-guided fractionation of plant extracts is a standard method for isolation of bioactive natural products. Especially in the area of antiparasitic agents, natural products have played a pivotal role in the past, and there is a continued interest in antimalarial activity of plants and plant isolates.^{40,41} Bioactivity-guided fractionation of extracts of *Z. multiflora*⁴² and *Z. vulgaris*⁴³ using *P. falciparum* growth inhibition assay led to isolation of the pentacyclic triterpenes **1** and **2**, which exhibited antiparasmodial activity as shown in Table 1.

In vitro antiparasmodial activity of **1** has been reported previously^{44,45} with IC_{50} values similar to those found in the present work (Table 1). The disclosure of the in vitro antiparasmodial activity of **1** has led to an evaluation of its in vivo effects using *Plasmodium berghei* murine malaria model.⁴⁵ However, no in vivo activity was found.⁴⁵ Very recently, the antiparasmodial activity of **1** was sought improved using a combinatorial synthesis approach.⁴⁶ However, these previous studies provided

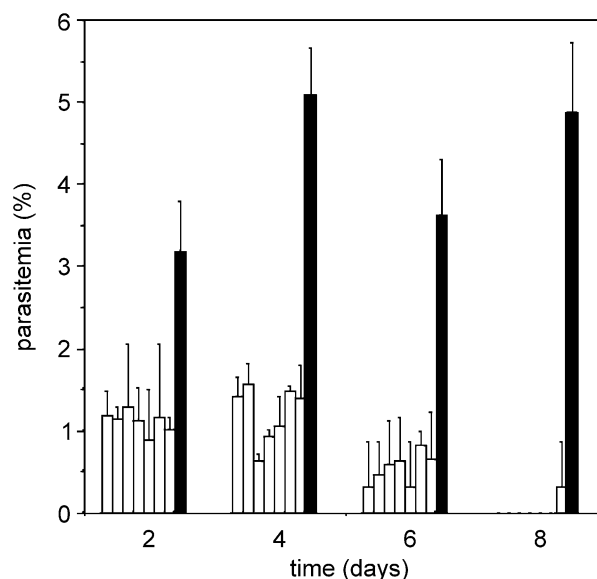


Figure 5. Growth of *Plasmodium falciparum* 3D7 in erythrocytes loaded with betulinic acid (**1**), betulinic aldehyde (**2**) and betulin (**4**). For each day, the columns represent from left to right: erythrocytes pre-incubated for 24 h with $50 \mu\text{g mL}^{-1}$ ($109.5 \mu\text{M}$) of **1**, $6.25 \mu\text{g mL}^{-1}$ ($13.7 \mu\text{M}$) of **1**, $50 \mu\text{g mL}^{-1}$ ($113.5 \mu\text{M}$) of **2**, $6.25 \mu\text{g mL}^{-1}$ ($14.2 \mu\text{M}$) of **2**, $200 \mu\text{g mL}^{-1}$ ($451.7 \mu\text{M}$) of **4**, $50 \mu\text{g mL}^{-1}$ ($112.9 \mu\text{M}$) of **4**, $6.25 \mu\text{g mL}^{-1}$ ($14.1 \mu\text{M}$) of **4** (white columns), and control (black column). The control was sub-cultured on day 4.

no clues about a possible mechanism of the in vitro antiparasmodial effect of **1**. We have now found, that **1** and its analogues **2** and **4–6** are potent membrane-active agents exerting their stomatocytogenic or echinocytogenic action in the concentration region, where the in vitro antiparasmodial effect is observed. This is similar to what was very recently reported for **3**²³ and the diterpene dehydroabietinol.²⁴

There are only minor variations in the in vitro antiparasmodial activity of **1–6** (Table 1). The observed erythrocyte membrane alterations correlated well with the antiparasmodial effect (Table 2). However, the effects on the membrane shape differed conspicuously in the series **1–6**. Thus, in spite of the closely related structures, compounds **2**, **3** and **5** caused formation of stomatocytes, whereas the formation of echinocytes was observed with **1**, **4** and **6** (Table 2, Figs 2–4). As expected, endo- or exovesiculation was observed with compounds that induce formation of stomatocytes or echinocytes, respectively (Figs 2–4). To our knowledge, this is the first demonstration of varying membrane

effects in a series of closely related compounds where a single functional group is being modified while keeping a large hydrocarbon skeleton unchanged. There is a clear structure–activity relationship in the observed membrane effects (Table 2). Thus, compounds **1**, **4** and **6** having a functional group at C-28 that is capable of acting as a hydrogen bond donor (COOH, CONH₂, CH₂OH) are echinocytogenic, while the presence at this position of a group that cannot donate a hydrogen bond (CH₃, CHO, COOCH₃) results in stomatocytogenic compounds **2**, **3** and **5** (Table 2). According to the classical bilayer couple theory,^{5,47,48} echinocytes are formed when a compound incorporates into and expands the outer leaflet of the membrane lipid bilayer, whereas stomatocytes are formed when the incorporation takes place predominantly into the inner leaflet. Thus, **1**, **4** and **6** are apparently incorporated mainly into the outer leaflet, and **2**, **3** and **5** into the inner leaflet. Previous studies have highlighted the importance of amphiphile charge for the type of membrane effect they induce.^{49–51} The present results emphasize the importance of hydrogen bonds in the interaction with the erythrocyte membrane. Since hydrogen bonding appears to be important for the interactions of cholesterol with membrane phospholipids,⁵² mainly with sphingomyelin present predominantly in the outer membrane leaflet, the triterpenoids may also interact with sphingomyelin. The biphasic behavior of **4** (Fig. 4) may be due to its redistribution into the inner leaflet at high concentrations, similarly as previously reported with some amphiphiles.⁵⁰

Loading the triterpenes **1**, **2** and **4** into the erythrocyte membrane, followed by removal of excess of the chemicals by washing, rendered the cells unsuitable for parasite growth, as illustrated in Figure 5. Thus, the presence of the compounds in the erythrocyte membrane and not in the growth medium is important for the inhibition of the parasite growth. It is evident that both the stomatocytogenic and the echinocytogenic compounds exhibit this effect. The observation of excess of extracellular merozoites in the cultures employing the membrane-modified erythrocytes suggests that the invasion was inhibited. The presence of extra-erythrocytic schizonts suggests that the parasites may have been expelled from the host cells, similarly as recently reported upon depletion of cholesterol.³¹

Pentacyclic triterpenes are ubiquitous dietary constituents and their interactions with cellular membranes may be responsible for various physiological and in vitro effects. Further studies are necessary to investigate the possible influence of pentacyclic triterpenes on the composition and function of lipid rafts, which were recently shown to play a key role in the erythrocyte invasion by merozoites.^{31,32}

4. Conclusion

The key observations reported here are: (1) that hydrogen bonding properties of pentacyclic triterpenes determine their mode of incorporation into the erythrocyte

membrane, and (2) that incorporation of the triterpenes into erythrocyte membrane is prohibitive with respect to malaria parasite invasion and growth irrespectively of the type of transformation they induce (formation of stomatocytes or echinocytes). Although the membrane changes observed by electron microscopy (Figs 2–4) are difficult to quantify, there is a very good qualitative correlation between the extent of membrane shape alterations and the observed IC₅₀ values for the inhibition of proliferation of malaria parasites. Erythrocyte membrane and the parasite vacuolization process might thus be a possible novel target⁵³ for antimalarial therapy, especially if a way of selective targeting of the infected erythrocytes can be found. The link between the in vitro antiplasmodial effect and the erythrocyte membrane modification has also an immediate significance with respect to interpretation of *Plasmodium* drug sensitivity assays employing intra-erythrocytic parasite stages. Moreover, compounds **1–6** provide a potentially useful model for studies of interaction of cholesterol analogues with lipid bilayers.⁵⁴

5. Experimental

5.1. General

NMR spectra were obtained on a Bruker AMX 400 or a Varian Gemini 2000 instrument (proton frequency 400.13 or 300.06 MHz, respectively), using tetramethylsilane (¹H and ¹³C) or trichlorofluoromethane (¹⁹F) as internal standard. Optical rotations were measured using a Perkin-Elmer 241 polarimeter. Merck silica gel 60 F₂₅₄ plates were used for thin layer chromatography (TLC), the spots being visualised by spraying with anisaldehyde-sulfuric acid reagent followed by heating. Vacuum liquid chromatographic (VLC) separations were performed on Merck silica gel 60H for TLC. Preparative HPLC separations were achieved on a chromatograph consisting of a Gynkotek P 580 pump, Rheodyne 7725 injector, Shimadzu SPD-10AV spectrophotometric detector operating at 205 nm, and a recorder, using a 25 cm×1.6 cm I. D. column packed with Lichrosorb Si-60 (7 μm) and eluted with heptane–EtOH 9:1 at 6 mL min^{−1}. Melting points were determined in capillaries and are uncorrected. Betulinic acid (**1**), lupeol (**3**) and betulin (**4**) were purchased from Sigma-Aldrich. All chemicals and materials were used as received. Elemental analyses were performed at the Department of Chemistry, H. C. Ørsted Institute, University of Copenhagen.

5.2. *P. falciparum* drug sensitivity assay

The assay was performed with *P. falciparum* strain 3D7 (initial parasitemia 1.5%) essentially as previously described.^{23,39} Briefly, the parasites were exposed to test compounds for 48 h in microtiter plates, measuring uptake of [³H]phenylalanine during the second half of this period. Extracts and fractions were tested at 12.5, 25, 50 and 100 μg mL^{−1}. Inhibition curves for pure compounds were constructed using 8–15 different concentrations, each tested in duplicate. IC₅₀ values were

derived from the radioactivity uptake curves by non-linear curve fitting with GraFit ver. 4.06 program from Erithacus Software Ltd., using four-parameter logistic equation. The reported values are averages of three independent determinations. Chloroquine diphosphate was used as a reference in all determinations.

5.3. Parasite growth in erythrocytes pre-treated with betulinic acid (1), betulinic aldehyde (2) and betulin (4)

Solutions of the test compounds (**1**, **2** and **4**) in 0.4 mL of DMSO were mixed with 38.6 mL of RPMI 1640 medium (containing all additives), 1 mL of packed human erythrocytes (10^{10} cells) was added, and the suspensions were incubated at 37 °C in an atmosphere containing 93% N₂, 5% CO₂ and 2% O₂. The final concentration of the compounds was 6.25 or 50 $\mu\text{g mL}^{-1}$, with an additional concentration of 200 $\mu\text{g mL}^{-1}$ for **4**. Reference flasks contained the same amount of DMSO but no test compounds. After a 24 h incubation period, the erythrocytes were separated (2000 rpm, 10 min) and washed three times with RPMI 1640 medium and once with the growth medium. The washing process was monitored by microscopy in order to exclude the possibility that cell shape changes occurred during this procedure. The washed cells were then used for sub-cultivation of *P. falciparum* 3D7 in 25 cm² flasks. Thus, 200 μL of the pre-incubated erythrocytes were mixed with 20 μL of parasitized erythrocytes (parasitemia 5%) and incubated with 5 mL of the growth medium. The medium was replaced with a fresh portion every 48 h. Every second day a small sample of the erythrocytes was withdrawn and parasite counts were determined microscopically, counting at least three microscopic fields with an average of 100 erythrocytes in each field. When parasitemia reached 5% (day 4, control culture), the cells were sub-cultured similarly as described above.

5.4. Erythrocyte membrane shape changes

Non-parasitized erythrocytes were incubated in 96-well microtiter plates in medium containing test compounds **1–6** as in the assay for antiplasmodial activity,^{23,39} but omitting the addition of [³H]phenylalanine. Each compound was tested in at least five concentrations around its IC₅₀ value (50, 12.5, 6.25, 3.13 and 0.78 $\mu\text{g mL}^{-1}$ for **1** and **2**; 200, 50, 25, 12.5 and 3.13 $\mu\text{g mL}^{-1}$ for **3**; 200, 50, 25, 6.25, 3.13, 1.56 and 0.39 $\mu\text{g mL}^{-1}$ for **4**; 25, 6.25, 3.13, 1.56 and 0.39 $\mu\text{g mL}^{-1}$ for **5**; 50, 12.5, 6.25, 3.13 and 0.78 $\mu\text{g mL}^{-1}$ for **6**). Reference wells contained erythrocytes in the medium alone or in the medium containing 2% of DMSO. After 48 h of incubation, 20 μL samples were spread on microscope slides, allowed to dry, fixed with methanol, and stained with Giemsa for phase-contrast light microscopy. Parallel samples were prepared for transmission electron microscopy as previously described.²³

5.5. Plant material

Aerial parts of *Zataria multiflora* Boiss., Lamiaceae (syn. *Zataria bracteata* Boiss.), and roots of *Zizyphus* (*Zizyphus*) *vulgaris* Lam., Rhamnaceae (syn. *Zizyphus*

jujuba Mill., *Zizyphus sativa* Gaertn.), were collected in Ardestan, Isfahan, Iran. Voucher specimens (Alt. 899 and Alt. 1058) were deposited in the herbarium of the Isfahan Research Centre of Natural Resources and Animal Science, Isfahan, Iran.

5.6. Isolation of betulinic acid (1) from *Z. multiflora* Boiss.

Dried and pulverised plant material (aerial parts, 100 g) was extracted with 3×1.5 L of 96% EtOH. The extract was evaporated, and the residue (9.4 g) fractionated on a VLC column, eluting with toluene followed by toluene–EtOAc 4:1, EtOAc, and MeOH, and testing the fractions for antiplasmodial activity. Active material (IC₅₀ < 12.5 $\mu\text{g mL}^{-1}$) eluted with EtOAc (1.8 g) was further fractionated by VLC using toluene, toluene–EtOAc 95:5, and toluene–EtOAc 1:1. Crystallisation from EtOAc of a fraction eluted with toluene–EtOAc 95:5 gave 24 mg (0.024%) of betulinic acid (**1**); $[\alpha]_{\text{D}}^{25} = +8^\circ$ (*c* 0.11, pyridine); ¹H and ¹³C NMR data identical with those of authentic material.

5.7. Isolation of betulinic acid (1) and betulinic aldehyde (2) from *Z. vulgaris* Lam.

Dried and pulverised plant material (root, 100 g) was extracted with 3×1 L of 96% EtOH, the extract was evaporated, and the residue (12.8 g) fractionated similarly as above, to give 27 mg (0.027%) of betulinic acid (**1**), $[\alpha]_{\text{D}}^{25} = +7^\circ$ (*c* 0.14, pyridine), and 6 mg (0.006%) of betulinic aldehyde (**2**); $[\alpha]_{\text{D}}^{25} = +16^\circ$ (*c* 0.14, CHCl₃); ¹H and ¹³C NMR spectra identical with those obtained with authentic samples (commercial **1** or synthetic **2**, see below).

5.8. Synthesis of betulinic aldehyde (2)

Betulin (**4**) (100 mg) was added to a vigorously stirred suspension of pyridinium dichromate (79 mg) in dry CH₂Cl₂ (8 mL).³³ After 0.5 h, an additional portion of **4** (100 mg) was added to the reaction mixture, which was then stirred for an additional 21.5 h. The mixture was filtered through a layer of silica gel (Merck 60H) on a 2-cm Büchner funnel, washing with EtOAc (75 mL). The filtrate was concentrated, the residue (0.19 g) was dissolved in hexane–CH₂Cl₂ (1:2, 8 mL), and the solution was applied to a VLC column (3×3 cm). Elution with hexane, and then with hexane–EtOAc (50:1 to 5:1), monitored by TLC and ¹H NMR, gave **2** (40 mg, 20%) in addition to unreacted **4** (82 mg, 41%) and products with 3-oxo group.³³ The aldehyde **2** was recrystallized from MeOH; white crystals, mp 183–187 °C, lit.³⁵ mp 192–193 °C; $[\alpha]_{\text{D}}^{25} = +18^\circ$ (*c* 0.38, CHCl₃), lit.³⁵ $[\alpha]_{\text{D}}^{25} = +19^\circ$; ¹H and ¹³C NMR data as reported.^{35,36}

5.9. Synthesis of betulinic acid methyl ester (5)

The ester **7** (104 mg) was treated with 1 M methanolic NaOMe (25 mL) in a closed steel vessel at 70 °C for 16 h. The mixture was neutralized with HOAc (about 1.2 mL), concentrated, and the residue partitioned between EtOAc and H₂O. The organic layer was separated,

washed with water, dried (Na_2SO_4) and evaporated. The residue was dissolved in CH_2Cl_2 and loaded onto a VLC column (3×3 cm), which was eluted with hexane and then with hexane–EtOAc (50:1 to 10:1), to yield the methyl ester **5** (56 mg, 82%). The ester **5** was recrystallized from MeOH; white needles, mp 217–220 °C, lit.³⁷ mp 224–225 °C; $[\alpha]_{\text{D}}^{25} = +5^\circ$ (*c* 0.17, CHCl_3), lit.³⁷ $[\alpha]_{\text{D}}^{25} = +4^\circ$; ^1H and ^{13}C NMR data as reported.³⁷

5.10. Synthesis of betulinic acid amide (**6**)

The ester **7** (108 mg) was treated with liquid NH_3 –THF (1:1, 30 mL) in a closed steel vessel at 55–60 °C for 3 days. After cooling to –78 °C the vessel was opened, ammonia was allowed to evaporate, and the solvent was removed in vacuo. The residue was dissolved in CH_2Cl_2 and loaded onto a VLC column (3×3 cm), which was eluted with hexane and then with hexane–EtOAc (5:1 to 5:2) to yield the amide **6** (66 mg, 93%), recrystallized from MeOH; white prisms, mp 254–257 °C (decomposition); $[\alpha]_{\text{D}}^{25} = +6^\circ$ (*c* 0.47, CHCl_3); ^1H NMR (CDCl_3): $\delta = 5.53$ and 5.35 (each 1H, br s, amide protons), 4.73 and 4.59 (each 1H, br s, olefinic protons), 3.18 (dd, 1H, $^3J_{3\text{ax},2\text{ax}} = 11.1$ Hz and $^3J_{3\text{ax},2\text{eq}} = 5.1$ Hz, H-3ax), 3.08 (dt, 1H, $^3J_{19\text{ax},18\text{ax}} = ^3J_{19\text{ax},21\text{ax}} = 11.1$ Hz, $^3J_{19\text{ax},21\text{eq}} = 4.4$ Hz, H-19ax) 1.68 (s, 3H, H-30), 0.97, 0.96, 0.96, 0.81, 0.75 (each 3H, s, H-23, H-24, H-25, H-26 and H-27), 2.50–0.66 (m, remaining protons); ^{13}C NMR (CDCl_3): $\delta = 178.9$ (C-28), 150.8 (C-20), 109.4 (C-29), 79.0 (C-3), remaining carbons: 55.9, 55.4, 50.7, 49.9, 46.6, 42.6, 40.8, 38.9, 38.8, 38.4, 37.7, 37.2, 34.4, 34.0, 30.8, 29.5, 28.0, 27.4, 25.6, 20.9, 19.5, 18.3, 16.2 (two carbons), 15.4, 14.6; elemental analysis: calcd (%) for $\text{C}_{30}\text{H}_{49}\text{NO}_3 \times 0.5\text{H}_2\text{O}$ C 76.53, H 10.84, N 3.01, found C 77.20, H 10.75, N 3.02.

5.11. Synthesis of betulinic acid pentafluorophenyl ester 3-O-trifluoroacetate (**7**)

Betulinic acid (**1**) (519 mg, 1.14 mmol) was dissolved in DMF (6 mL), and pentafluorophenyl trifluoroacetate (588 μL , 3×1.14 mmol) and pyridine (201 μL , 2.2×1.14 mmol) were added. The mixture was stirred for 3.5 h, when it was diluted with EtOAc (100 mL), and washed successively with 0.1 M HCl, saturated aqueous NaHCO_3 and water (each with 2×80 mL). The organic layer was dried (Na_2SO_4) and concentrated. The residue was dissolved in CH_2Cl_2 and purified repeatedly on a VLC column (5×5 cm), eluting with hexane and hexane–EtOAc (100:1), which afforded **7** (743 mg, 76%) as a white foam, recrystallized from ether–pentane; mp 176–177 °C; $[\alpha]_{\text{D}}^{25} = -4.5^\circ$ (*c* 0.37, CHCl_3); ^1H NMR (CDCl_3): $\delta = 4.74$ and 4.63 (each 1H, br s, olefinic protons), 4.67 (dd, 1H, $^3J_{3\text{ax},2\text{ax}} = 11.0$ Hz and $^3J_{3\text{ax},2\text{eq}} = 5.2$ Hz, H-3ax), 2.96 (dt, 1H, $^3J_{19\text{ax},18\text{ax}} = ^3J_{19\text{ax},21\text{ax}} = 11.1$ Hz, $^3J_{19\text{ax},21\text{eq}} = 4.9$ Hz, H-19ax) 1.71 (s, 3H, H-30), 1.01, 0.96, 0.89, 0.89, 0.78 (each 3H, s, H-23, H-24, H-25, H-26 and H-27), 2.51–0.81 (m, remaining protons); ^{13}C NMR (CDCl_3): $\delta = 172.1$ (C-28), 175.4 (q, $^2J_{\text{C},\text{F}} = 41.5$ Hz, CO of the trifluoroacetyl group), 149.7 (C-20), 141.3 (dd, $^1J_{\text{C},\text{F}} = 251$ Hz, $^2J_{\text{C},\text{F}} = 12.5$ Hz, C-2'), 139.3 (dt, $^1J_{\text{C},\text{F}} = 252$ Hz, $^2J_{\text{C},\text{F}} = 14$ Hz, C-4'), 137.9 (dt, $^1J_{\text{C},\text{F}} = 252$ Hz, $^2J_{\text{C},\text{F}} = 14$ Hz, C-3'), 125.3 (t,

$^2J_{\text{C},\text{F}} = 14.5$ Hz, C-1'), 114.7 (q, $^1J_{\text{C},\text{F}} = 286$ Hz, CF_3), 110.2 (C-29), 86.3 (C-3), remaining carbons: 57.7, 55.4, 50.5, 49.5, 46.7, 42.5, 40.7, 38.4, 38.3, 38.11, 37.13, 37.0, 34.2, 31.8, 30.3, 29.7, 27.8, 25.5, 23.3, 20.9, 19.4, 18.1, 16.2 (two carbons), 15.8, 14.7; ^{19}F NMR (CDCl_3): $\delta = -75.8$ (s, 3 F, CF_3), -153.7 (m, 2 F, F-2'), -158.8 (t, 1 F, F-4'), -163.0 (m, 2 F, F-3'); elemental analysis: calcd (%) for $\text{C}_{38}\text{H}_{46}\text{F}_8\text{O}_4$ C 63.50, H 6.45, found C 63.43, H 6.41.

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